

CORSO INTEGRATO DI GENETICA  
AA 2011/12

Prof Alberto Turco

Martedì 18.10.11  
Lezioni 13 e 14

Trattamento malattie genetiche  
Terapia genica

Distinguere:  
trattamento di una malattia genetica da  
trattamento genetico di una malattia

...Malattie genetiche intrattabili?

No! PKU, sordità, emofilia....

NB: Non c'è correlazione tra “causa” e “trattabilità”



# X Congresso Nazionale SIGU

14 - 16 novembre 2007  
17 novembre 2007 • Corsi di Aggiornamento  
Palazzo dei Congressi, Montecatini Terme (PT)

PROGRAMMA DEFINITIVO



10 anni 1997-2007

## PROGRAMMA SINTETICO

Mercoledì, 14 novembre 2007

9.00 - 13.00

Registrazione e iscrizioni  
Affissione Poster

15.00 - 16.00

**INAUGURAZIONE DEL CONGRESSO** (Auditorium Sala Elio)  
Franca Dagna Bricarelli, Presidente SIGU

16.00 - 18.00

**Sessione Plenaria** (Auditorium Sala Elio)  
**TERAPIA FARMACOLOGICA PER LE MALATTIE GENETICHE**  
Moderatori: Maja Di Rocco (Genova), Romano Tenconi (Padova)

16.00

Mitocondri e collagene VI. Dal modello animale ad una terapia farmacologica della distrofia muscolare congenita di Ullrich  
Paolo Bernardi (Padova)

16.30

Prospettive terapeutiche per le laminopatie  
Giuseppe Novelli (Roma)

17.00

Nuove prospettive terapeutiche per la sindrome di Marfan  
Eloisa Arbustini (Pavia)

17.30

Nuove terapie farmacologiche per le malattie lisosomiali; riduzione del substrato e chaperones.  
Giancarlo Parenti (Napoli)

18.00 - 18.45

**SALUTO DELLE AUTORITÀ** (Auditorium Sala Elio)  
E' stato invitato a partecipare il Ministro della Salute, Livia Turco

# **Trattamento “convenzionale” malattie genetiche**

(le tre “R” = Restriction, Replacement, Removal)

## **Restriction – Limitazione di substrati**

Es Fenilalanina – Fenichetonuria

Colesterolo – Ipercolesterolemia

## **Replacement – Sostituzione di prodotto deficitario/tessuto**

Es Fattore VIII – Emofilia A

Enzimi digestivi – Fibrosi cistica

Ormoni tiroidei – Ipotiroidismo

Trapianto di cuore, rene, fegato

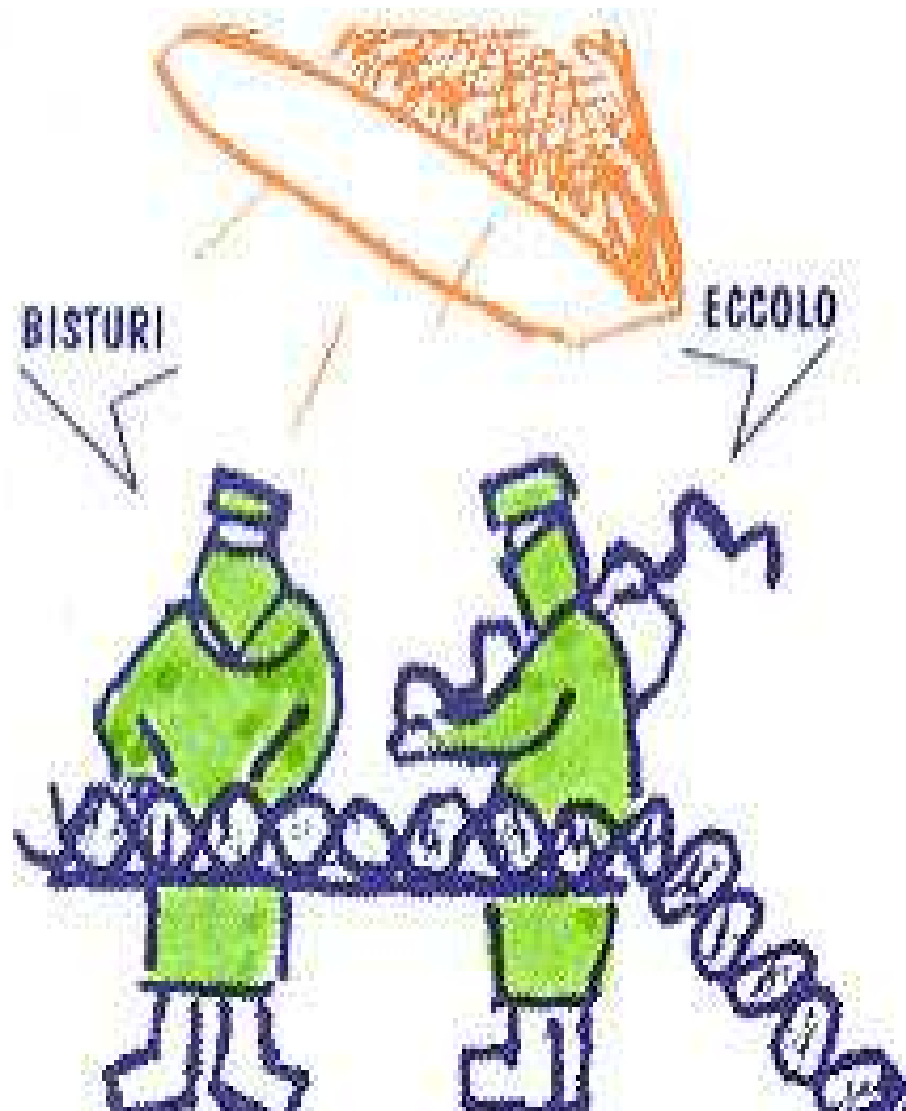
## **Removal – Rimozione di metaboliti tossici o tessuti malati**

Es Salassoterapia – emocromatosi (accumulo di ferro)

Penicillamina (chelante del rame) – m.di Wilson

Colectomia (asportazione colon) – Poliposi colica adenomatosa

# Terapia genica.....



# TERAPIA GENICA

## Definizione

**Introduzione (mediante vettore, es virus) di sequenze di acido nucleico ricombinante nelle cellule (somatiche o [?] germinali) di un paziente (o di un embrione ?...figlio?.....atleta?.....)**

## Obiettivi

**Aggiungere, riparare(?) o bloccare la funzione e/o l'espressione di specifici geni nel trattamento di malattie genetiche, ereditarie e non ereditarie (o di condizioni non patologiche....? Altezza, appetito, massa muscolare, memoria.....?)**

# Tipi di terapia genica

1. **TG ex vivo**: le cellule bersaglio sono rimosse dal paziente, modificate geneticamente in vitro e reintrodotte
2. **TG in vivo**: introduzione diretta del “gene terapeutico” nel paziente (iniezione, inalazione)



# IX Congresso Nazionale SIGU

8-10 novembre 2006

11 novembre 2006 > Corsi di aggiornamento

Palazzo del Cinema, Lido di Venezia

## PROGRAMMA



15.15-17.00 **SESSIONE PLENARIA** (Sala Grande)

### **Terapia Genica**

Moderatori: A. Cao (Cagliari), A. Colosimo (Teramo)

15.15 **Terapia genica dell'ADA-SCID**  
A. Aiuti (Milano)

15.50 **La terapia genica e cellulare della Fibrosi Cistica**  
M. Conese (Milano)

16.25 **Globin gene transfer for the treatment of severe hemoglobinopathies**  
M. Sadelain (New York, USA)

17.00-17.30 **Premio Giuseppe Pilia per le malattie complesse** (Sala Grande)  
**Premi SIGU a giovani ricercatori** (Sala Grande)

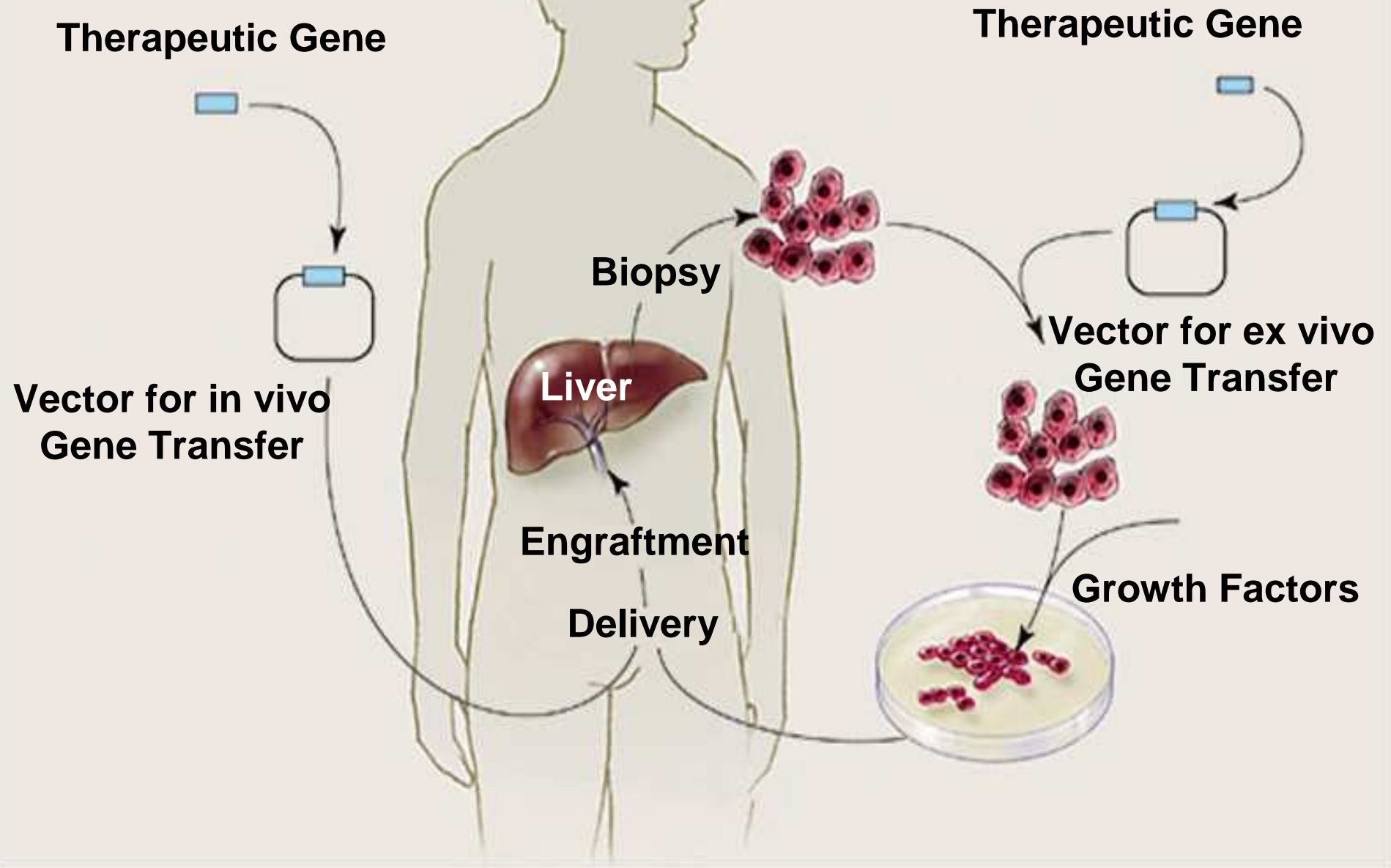
17.30 **CHIUSURA DEL CONGRESSO** (Sala Grande)

18.00 **Test di valutazione dell'apprendimento** (Sala Grande)



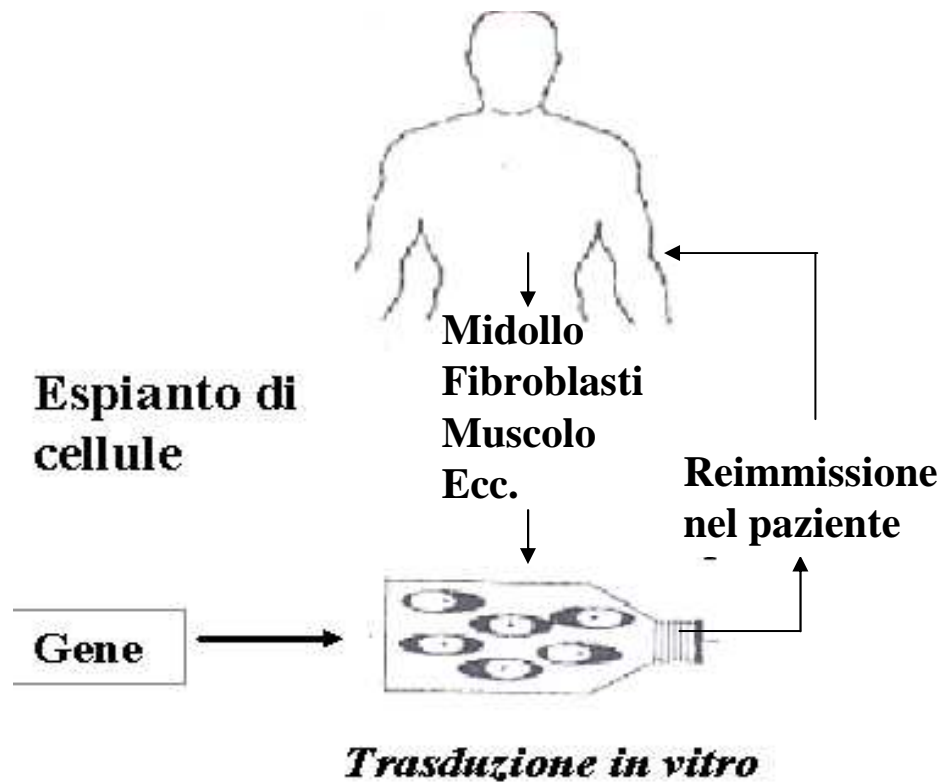
## ***In vivo*** **Gene Therapy**

## ***Ex vivo*** **Gene Therapy**

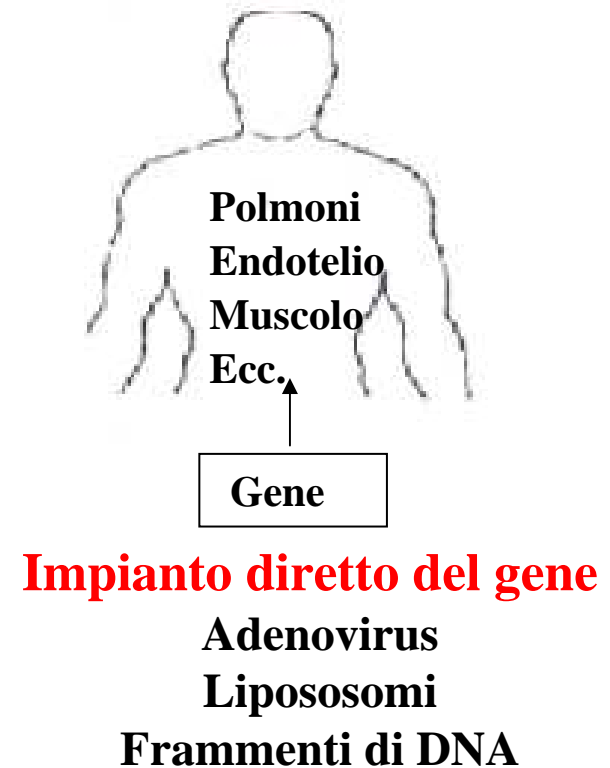


# TERAPIA GENICA

## Ex vivo



## In vivo



# TERAPIA GENICA

- SOMATICA

- GERMINALE

- MIGLIORATIVA

# **TIPI DI T.G. SOMATICA**

## **1. Gene supplementation (augmentation, GAT)**

TG “classica” sostitutiva – loss of function  
(es.CF, emofilia)

## **2. Gene replacement**

(gain of function, es. malattie dominanti)

## **3. Inibizione mirata dell'espressione genica**

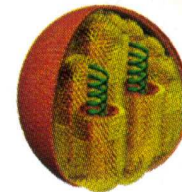
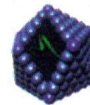
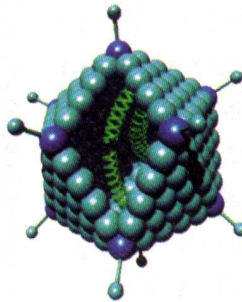
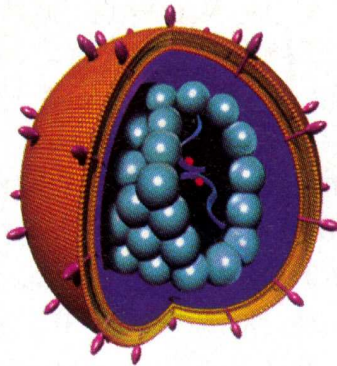
(es TG antisenso, RNAinterference)

# **TERAPIA GENICA: VETTORI**

- **VETTORI VIRALI:** RETROVIRUS  
ADENOVIRUS  
VIRUS ADENO-ASSOCIATI  
HERPES SIMPLEX VIRUS  
LENTIVIRUS
- **VETTORI NON VIRALI:** PLASMIDI  
LIPOSOMI  
CONIUGATI DNA-PROTEINE

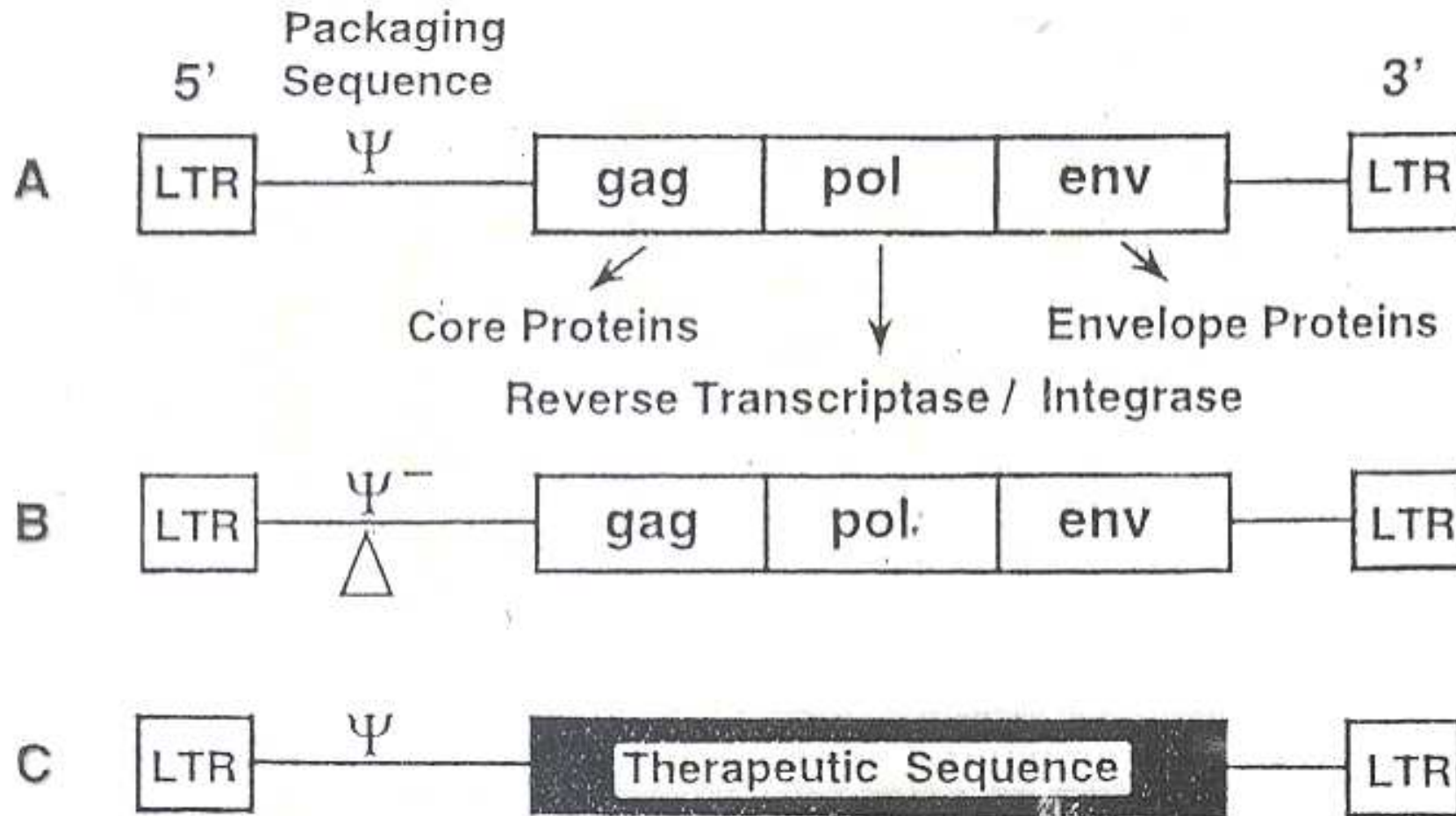


# VETTORI PER LA TERAPIA GENICA



	Retrovirus	Adenovirus	Virus adeno- -associati	Liposomi	DNA «nudo»
<b>Alcuni potenziali vantaggi</b>	Integrano i geni nei cromosomi dell'ospite, consentendo una stabilità a lungo termine	La maggior parte non causa gravi malattie; possono accogliere geni estranei di grandi dimensioni	Integrano i geni nei cromosomi dell'ospite; non causano malattie umane note	Non hanno geni virali e pertanto non causano malattie	Come i liposomi; si prevede che sia utile per le vaccinazioni
<b>Alcuni difetti dei vettori esistenti</b>	I geni si integrano a caso, pregiudicando a volte i geni dell'ospite; molti infettano solo cellule in divisione	I geni a volte funzionano transitoriamente, per la mancata integrazione o l'attacco del sistema immunitario	Non possono accogliere geni estranei di grandi dimensioni	Sono meno efficienti dei virus nel trasferire geni alle cellule	È inefficiente nel trasferimento genico e instabile in gran parte dei tessuti dell'organismo

# RETROVIRUS





# TERAPIA GENICA SOMATICA

## UNA DELLE PRIME APPLICAZIONI NELL'UOMO

### Correction of ADA-SCID by Stem Cell Gene Therapy Combined with Nonmyeloablative Conditioning

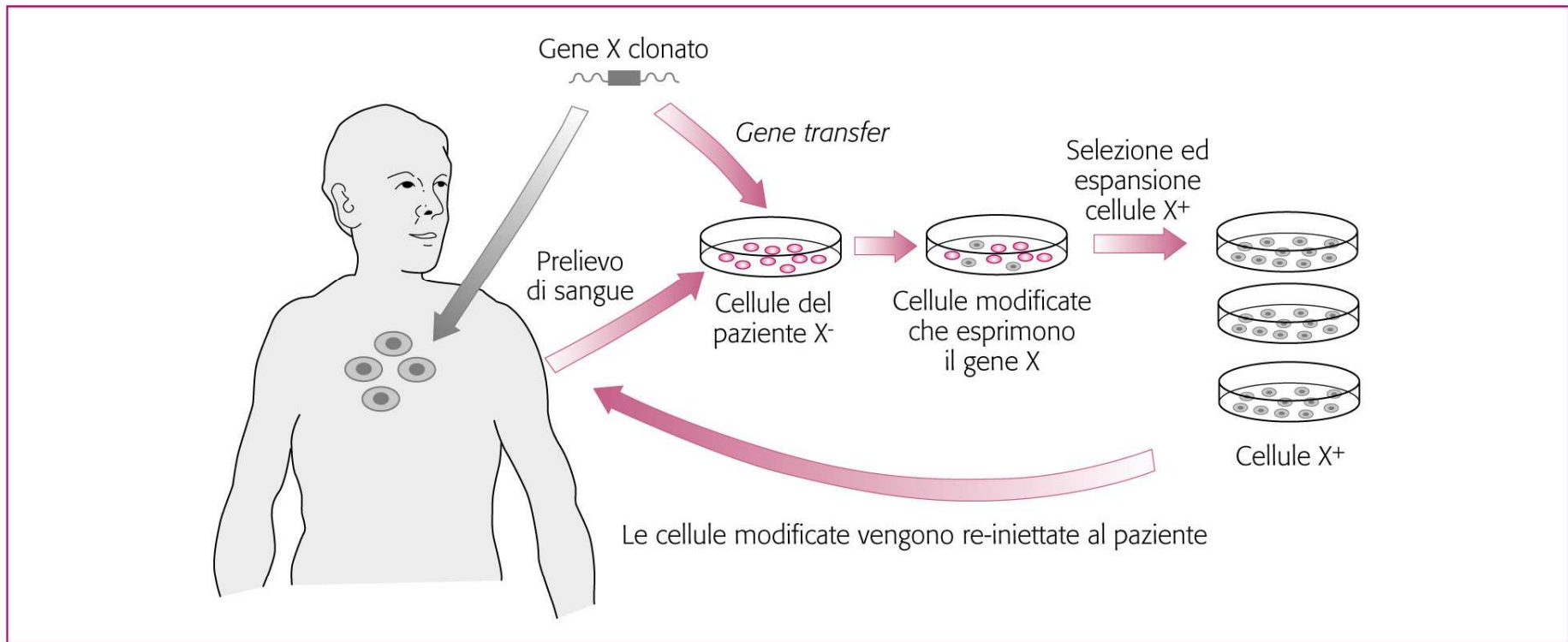
Alessandro Aiuti,<sup>1</sup> Shimon Slavin,<sup>2</sup> Memet Aker,<sup>2</sup>  
Francesca Ficara,<sup>1</sup> Sara Deola,<sup>1</sup> Alessandra Mortellaro,<sup>1</sup>  
Shoshana Morecki,<sup>2</sup> Grazia Andolfi,<sup>1</sup> Antonella Tabucchi,<sup>3</sup>  
Filippo Carlucci,<sup>3</sup> Enrico Marinello,<sup>3</sup> Federica Cattaneo,<sup>1</sup>  
Sergio Vai,<sup>1</sup> Paolo Servida,<sup>4</sup> Roberto Miniero,<sup>5</sup>  
Maria Grazia Roncarolo,<sup>1,6</sup> \* Claudio Bordignon<sup>1,6\*†</sup>

*Science, Giugno 2002*

ADA = Adenosina deaminasi

SCID = Severe Combined Immuno Deficiency





**Figura 11.12** Approccio *ex vivo* e *in vivo* di terapia genica. Nell'approccio *ex vivo*, alcune cellule di un paziente che presenta un deficit del gene X, vengono prelevate, il difetto genetico viene corretto *in vitro* e, successivamente, le cellule vengono reintrodotte nel paziente. Nell'approccio *in vivo*, il vettore di terapia genica viene iniettato direttamente nel paziente. (Modificata da Strachan T, Read AP. *Human molecular genetics*, 3<sup>rd</sup> ed. Garland Science, New York-London, 2003.)

# TERAPIA GENICA UNA STORIA CONTROVERSA

## Harmful potential of viral vectors fuels doubts over gene therapy

Erika Check, Washington

The troubled field of gene therapy was dealt a fresh blow this week, after a study suggested that modified viruses used in some trials might cause health problems.

The study, led by geneticist Mark Kay at Stanford University, California, examined a modified virus used in gene-therapy trials to treat haemophilia and cystic fibrosis. It revealed that the virus has the potential to cause the same problems that led to cancer in an unrelated gene-therapy trial last year.

In gene therapy, doctors use a gutted virus as a 'vector' to transfer corrective genes into a patient's cells. But if the vector stitches itself into a cell's genes, it can cause the cell to mutate and become cancerous. This was demonstrated last year, when two children who had gene therapy for severe combined immunodeficiency disease (SCID) developed leukaemia (see *Nature* 419, 545-546; 2002).

Scientists are still trying to establish exactly why the SCID patients developed cancer, and will discuss the trial at this week's meeting of the American Society of Gene



Doctors at Stanford treat a haemophilic in a gene-therapy trial — but how safe is the procedure?

2003



GENE THERAPY

## Second Child in French Trial Is Found to Have Leukemia

Febbraio 2005

## Second cancer case halts gene-therapy trials

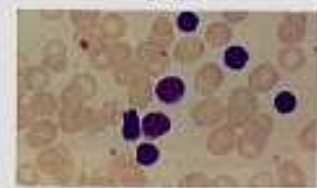
Erika Check, Washington

The world of gene therapy was shaken last year when a child treated in a French trial developed leukaemia. Researchers had pinned their hopes on this being an unfortunate one-off. Now those hopes have been dashed with the emergence of a second, almost identical case that could jeopardise the future of gene therapy.

The latest case centres on a three-year-old boy treated in a gene-therapy trial led by Alain Fischer at the Necker Hospital for Sick Children in Paris. Just under three years

ago, the world's only true success, Fischer has so far cured nine boys — including the two who now have leukaemia — out of 13 patients. And when the first child was diagnosed with cancer, some argued that it was an isolated event (see *Nature* 420, 595; 2002).

But Christof von Kalle of the Cincinnati Children's Hospital says analysis of the two boys' cells shows that the same molecular events probably caused the cancers. In both cases, the retroviral vector used to deliver the corrective gene has integrated itself into a stretch of DNA near a gene called *MLV*.



Blind cancer leukaemia has been detected in two patients who have received gene therapy.

Small but important: researchers hope that changes to a gene vector will reduce risks to patients.

## Gene therapy put on hold as third child develops cancer

## Gene therapists hopeful as trials resume with childhood disease

Erika Check, Minneapolis

A French gene-therapy trial that cured nine children of a severe disease, but gave two of them cancer, looks set to restart after a 22-month suspension.

The trial involves children who suffer from severe combined immunodeficiency disease (SCID). These children lack innate defences against infections and without treatment they can only survive in isolated environments. One US gene-therapy trial for the disease has also restarted, and others are likely to resume this year.

The suspension of the trial had deeply shaken the gene-therapy field, because SCID was the only disease that had ever been cured by such therapy. Researchers at the annual meeting of the American Society of Gene Therapy in Minneapolis, Minnesota, last week saw the resumption of the SCID trials as a bright spot after a long dark spell for the field.



Beyond the veil: Donald Kohn is one of those hoping to cure infants who are unable to fight infections.





**All change**  
Africa pressed to  
switch malaria  
drugs, despite cost  
p588



**Drug bust**  
Pharmaceutical firm  
targeted by New York  
attorney-general  
p589



**Lack of porpoise**  
Mexican fishermen's  
nets endanger rare  
marine mammal  
p590



**Fair funds**  
Economists urge  
redistribution of  
development aid  
p592

## Gene therapists hopeful as trials resume with childhood disease

Erika Check, Minneapolis

A French gene-therapy trial that cured nine children of a severe disease, but gave two of them cancer, looks set to restart after a 22-month suspension.

The trial involves children who suffer from severe combined immunodeficiency disease (SCID). These children lack innate defences against infections and without treatment they can only survive in isolated environments. One US gene-therapy trial for the disease has also restarted, and others are likely to resume this year.

The suspension of the trials had deeply shaken the gene-therapy field, because SCID was the only disease that had ever been cured by such therapy. Researchers at the annual meeting of the American Society of Gene Therapy in Minneapolis, Minnesota, last week saw the resumption of the SCID trials as a bright spot after a long dark spell for the field.

Specialists say there is still a risk that some children will develop cancer during the trials. But they say the trials should proceed, because the French technique has cured many children who suffer from the devastating illness.

"We're moving forward," says Donald Kohn, past president of the American Society of Gene Therapy and leader of one of the US trials. "No therapy is without risk, and now that we've had time to look back, we realize that this therapy even with the risk may be better than the current treatment," Kohn says.

The children in the French trial suffer from a version of the disease called X-linked SCID. For X-linked SCID patients, the alternative to gene therapy is a bone-marrow transplant. But these transplants are successful in only 70% of children, unless they have a suitable bone-marrow donor. Out of 18 children treated using gene therapy, 15 appear to have been cured of X-linked SCID.

The French trial, led by Alain Fischer of the Necker Hospital in Paris, was the first to show that infants could be cured through doses of a gene to correct their genetic deficiency. But in September 2002, Fischer announced that he had halted his trial because one of the participants had developed leukaemia. Another



Beyond the veil Donald Kohn is one of those hoping to cure infants who are unable to fight infections.

child came down with leukaemia a few months later. Both are alive and recovering from their cancers.

Fischer's announcement prompted the US Food and Drug Administration (FDA) to stop three gene-therapy trials in America — two in X-linked SCID, and one in another form of the disease called ADA-SCID. But an X-linked SCID study in Britain was allowed to continue, and seven children have now been treated in that study. Claudio Bordignon of the San Raffaele Telethon Institute for Gene Therapy in Milan, Italy, was also allowed to treat patients with ADA-SCID for whom other therapies had failed. Five patients have been treated in that trial, and not one has developed cancer.

The first SCID study to be resumed in the United States is led by Harry Malech and Jennifer Puck of the National Institutes of Health at Bethesda, Maryland. They were cleared to begin their trial in December and treated one child with X-linked SCID in January. So far, his condition is stable, Malech says.

The other two US trials are led by Kohn and by Kenneth Weinberg, both of the Children's Hospital Los Angeles. Both say that they are consulting with the FDA and hope to resume their trials later this year.

Since 2002, scientists have learned more about why gene therapy caused the X-linked SCID patients to get cancer. Such patients receive a copy of a gene they lack, called the *gamma-C* gene. This gene allows their immune cells to grow normally. But in the children who get leukaemia, *gamma-C* seems to switch on a cancer-causing gene called *LMO2*, which is found in human DNA.

Fischer and other scientists will adjust their treatment plans to minimize risks from this switching effect. For instance, in most cases Fischer will now only treat children older than 6 months, because they might be less vulnerable to cancer than the very young babies who developed cancer in his trial. Fischer will also place an upper limit on the number of corrected cells he injects into the children.

Some researchers take a different tack when balancing the risk of cancer against potential cure. Weinberg is asking the FDA to allow him to resume his X-linked SCID trial without limiting the age of the children enrolled or the dose of cells they receive. But like all other leaders of SCID trials in the United States, Weinberg will monitor each of his patients for signs of cancer for at least a decade after the trial.



Small but important: researchers hope that changes to a gene vector will reduce risks to patients.

## Gene therapy put on hold as third child develops cancer

Erika Check, Washington

Scientists have halted clinical trials of gene therapy to treat a rare immune disorder — less than a year after the trials were relaunched following an earlier stoppage.

The trials use gene therapy to treat different forms of severe combined immunodeficiency disease (SCID). The first trial to be stopped was halted in October 2002, and other trials were halted three months later, after two children in the trials developed cancer. But authorities allowed them to resume during the past year because the treatment had cured many children who lack reliable alternative treatments.

Researchers have now halted the trials again, after a third patient was found to have developed cancer. The suspension is a significant setback for the nascent field of gene therapy, because SCID treatment has been its most promising application to date.

The child with cancer was a patient of Alain Fischer of the Necker Hospital in Paris. He has been using gene therapy to treat the X-linked form of SCID, which is otherwise only treatable with bone-marrow transplant and is still often fatal. Fischer's trial restarted last May, and his team has treated one child since then.

But on 24 January, the French medical

regulatory authority AFSSPS announced that a child who was treated by Fischer in April 2002 now has cancer.

As a result, Fischer's trial and similar ones in the United States have been halted again. The agency also said that one of the original two patients who had been diagnosed with cancer — both of whom were in Fischer's trial — died last October.

Fischer is now investigating why the third child, who was treated at a later age than the previous two children, developed cancer. The child's cells did not seem to have the same genetic glitch that caused the first two cancers, he says, but he cautions that the analysis is still under way.

Fischer adds that he still believes in gene therapy as a treatment for X-linked SCID, because 15 children treated in this way are still alive, and 14 are doing well four years later. But his group will not treat any more children using its current gene-therapy system, he says. He adds that he plans to change a key step in the treatment by changing the vector — the modified virus that delivers the therapeutic gene to the patients.

"The efficacy is there, but we have to improve on the safety," Fischer says, adding that this is "not an uncommon situation" in medical research.





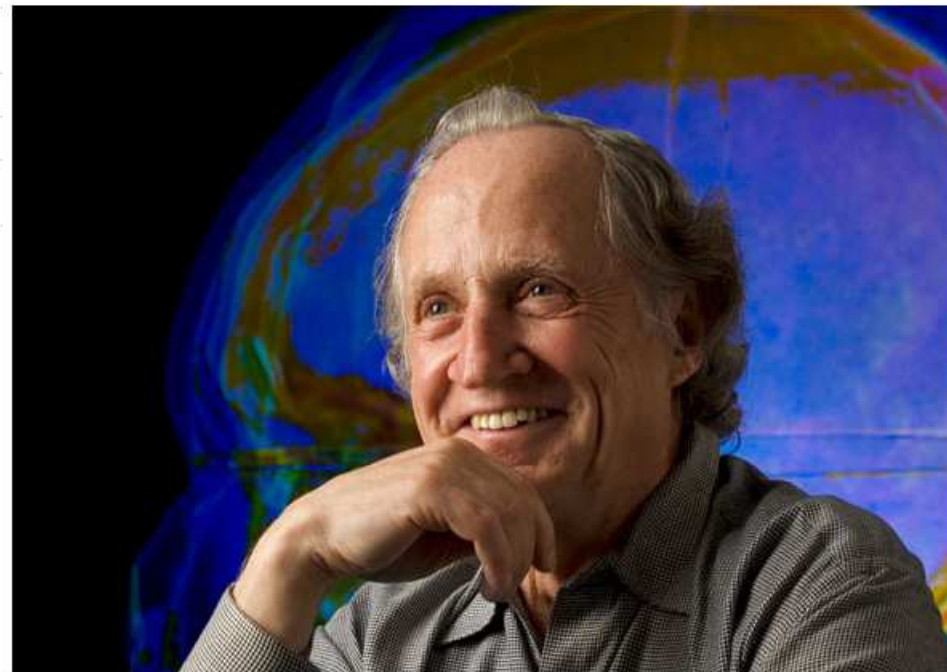
Academics and  
Research

▼ University Health Care ▼ The University of U

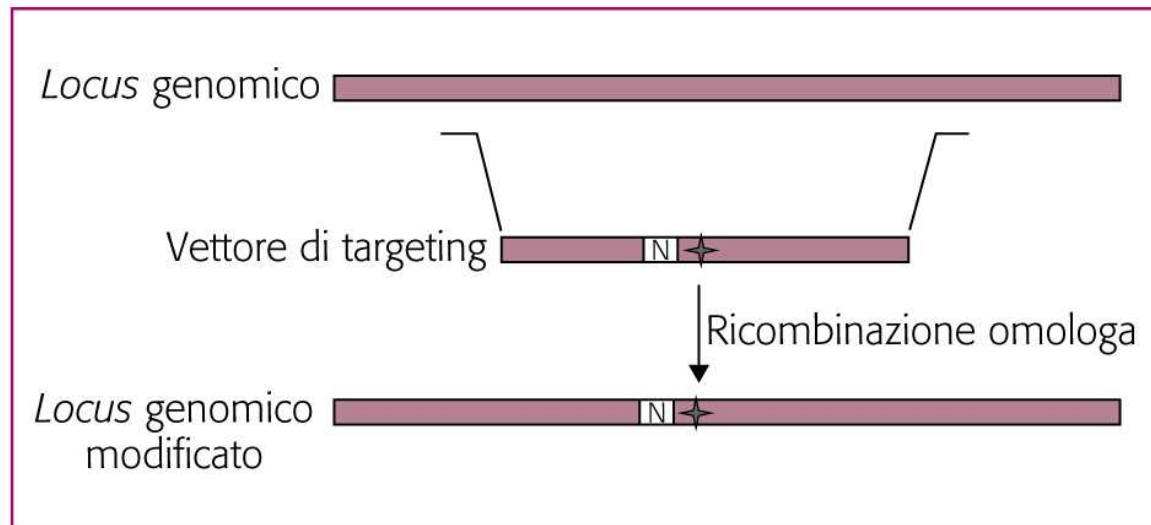
- ▶ News Releases
- ▶ Spotlight
- ▶ Grand Rounds Calendar
- ▶ Publications
- ▶ Contact Us
- ▶ Other University Public Relations

*U's Distinguished Professor Mario Capecchi Wins Nobel Prize for Physiology or Medicine*

# Nobel 2007 per la Medicina Mario Capecchi



# GENE TARGETING



**Figura 11.7** Rappresentazione schematica di un esperimento di gene targeting. Il vettore di targeting è un plasmide contenente circa 10 kb di DNA identico alla regione che viene sostituita, salvo che per la mutazione che si vuole inserire (la stella) e il gene di resistenza a un antibiotico, la neomicina (N), per permettere la selezione delle cellule ricombinanti. Alla fine del processo il *locus* genomico conterrà esclusivamente la modifica inserita dal vettori di targeting.

ESSAY

## Gene targeting in mice: functional analysis of the mammalian genome for the twenty-first century

Mario R. Capecchi

**Abstract** | Gene targeting in mouse embryonic stem cells has become the 'gold standard' for determining gene function in mammals. Since its inception, this technology has revolutionized the study of mammalian biology and human medicine. Here I provide a personal account of the work that led to the generation of gene targeting which now lies at the centre of functional genomic analysis.

Gene targeting — creating designed genomic modifications — has three enormous advantages relative to other procedures for introducing mutations into mice. First, the investigator chooses which genetic locus to mutate. Second, the technique takes full advantage of all the resources provided by the known sequences of the mouse and human genomes and, third, the investigator has complete control of how to modulate the chosen genetic locus<sup>1</sup>. This last advantage provides the investigator with the ability to design the genetic modification of the chosen locus so as to best address the specific biological question that is being pursued. Such modifications could include the creation of null mutations or hypomorphic mutations, the introduction of reporter genes to follow gene expression or determine cell lineage, and/or manipulation to restrict the effects of the mutation to any desired group of cells or organs (spatial restriction) or to any chosen temporal period during the life history of the mouse (temporal restriction). Surprisingly,

20 years after its development, the level of sophistication of genomic manipulations that are currently feasible in the mouse through gene targeting can still only be matched in far simpler organisms, such as bacteria and yeast.

Some investigators have questioned whether such reductionist approaches, which involve inferring gene function from the perturbations of a normal phenotype that are induced by the targeted mutations in one or a small number of genes, have sufficient power to provide significant understanding of how truly complex biological phenomena such as higher cognitive functions are mediated, particularly in an organism as complex as the mouse. Frankly, on more gloomy days, I sometimes raise similar questions myself. However, I am not aware of any other more successful means of dissecting complex biological phenomena into manageable, understandable components. It is to be hoped that through the summation of numerous such components, the desired level of clarity of even very complex biological phenomena will be achieved. Furthermore, when more holistic approaches have been applied to the analysis of the same processes, they have so far failed even more miserably, in my view, to provide significant understanding of these complex topics.

The initial development of gene targeting in mice required the solution to two basic problems. The first and foremost was how to produce specific mutations in a chosen gene

in cultured mammalian cells. The second was how to transfer this genetic modification to the mouse germline. Oliver Smithies' laboratory and mine worked independently on solutions to the first problem. Martin Evan's laboratory provided us with an approach for a solution to the second problem. What follows is a description of my laboratory's contributions to the development of gene targeting in the mouse. It is not meant to be comprehensive; it is rather a more personal description of our contributions to this field.

### 1977–1980: homologous recombination

The discoveries that directed my attention to the development of gene targeting began in 1977. At that time, I was exploring whether I could introduce DNA into nuclei of mammalian cells using extremely small glass needles (with tip diameters of less than one micron). Wigler and Axel had just demonstrated that mammalian cells deficient in thymidine kinase (*tk*<sup>-</sup>) could be transformed into *tk*<sup>+</sup> cells by exposing these cells to a DNA calcium phosphate co-precipitate containing the herpes virus thymidine kinase (*HSV-tk*; also known as *HHVAgp124*) gene<sup>2</sup>. Although this was an important advance for the field of somatic cell genetics, their protocol was not very efficient. With their procedure, incorporation of functional copies of the *HSV-tk* gene occurred in approximately one per million cells exposed to the DNA calcium phosphate co-precipitate. Using a similar selection procedure, I asked whether I could introduce functional copies of the *HSV-tk* gene into mouse *tk*<sup>-</sup> fibroblasts using very fine glass needles to inject the DNA directly into their nuclei<sup>3</sup>. This procedure proved to be extremely efficient. One cell in three that received the DNA stably passed the functional *HSV-tk* gene to its daughters. One does not often observe an almost 10<sup>6</sup>-fold improvement in the efficiency of a process. I first reported these results at a workshop organized by Frank Ruddle in 1978, held in Estarreja, Portugal. The extremely high efficiency of DNA transfer by microinjection made it practical for investigators to use this procedure to generate transgenic mice that contain random insertion of exogenous DNA. This was accomplished by microinjecting the desired DNA into nuclei of 1-cell mouse zygotes and allowing these embryos to come to term after surgical transfer to foster mothers<sup>4–6</sup>. Following this workshop, Frank Ruddle rapidly championed our results throughout the mouse research community.

The efficient transfer of the *HSV-tk* gene into cells by microinjection required the

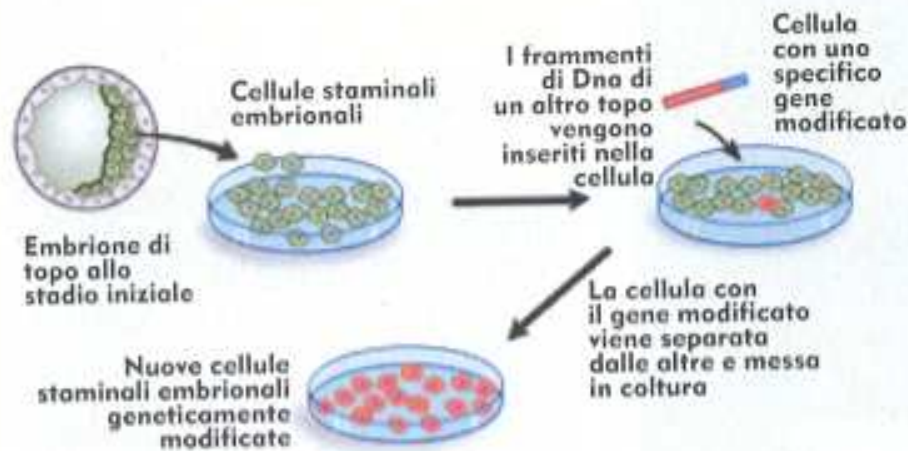
### PERSPECTIVES





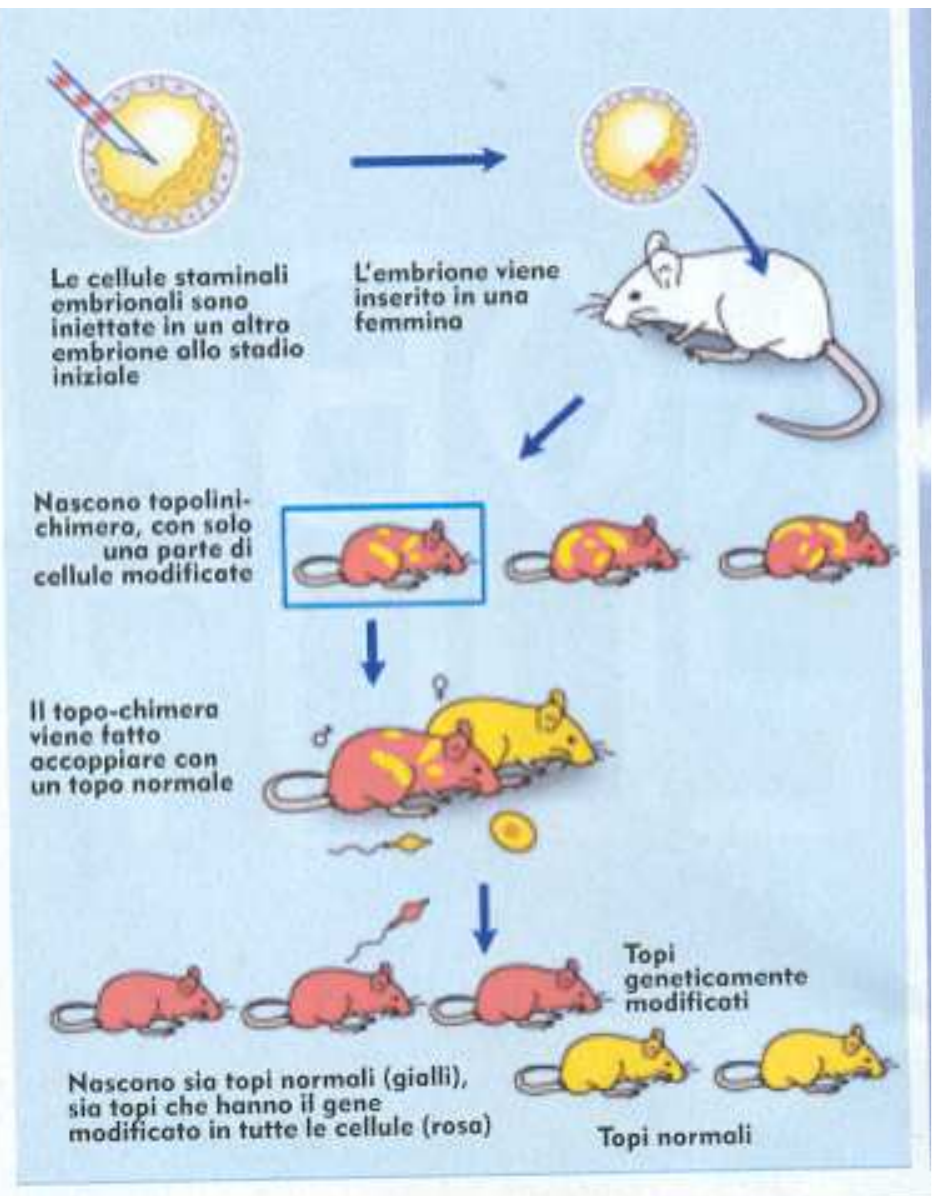
nella nostra vita quotidiana, trasformandola e determinando il progresso della nostra civiltà. Il premio Nobel ci consente di dare un nome e un volto alle persone che dovremmo ricordare e ringraziare, nel momento in cui potremo memorizzare centinaia di foto in una capocchia di spillo o curare malattie impossibili grazie al lavoro di Mario Capecchi e dei suoi colleghi sulle cellule staminali.

Una ricerca giustamente celebrata con tanti applausi allo «scienziato italo-americano» da molti politici italiani. Gli stessi che l'hanno vietata per legge nel nostro Paese.



## COSÌ SI MODIFICA IL GENE E SI OTTENGONO TOPOLINI MUTATI

Nel disegno qui sopra è rappresentata la strategia generale del gene targeting, ovvero la modificazione specifica di un gene. Si coltivano cellule staminali embrionali prelevate da un embrione di topo allo stadio iniziale (blastocisti); si inserisce in una cellula un frammento di Dna che contiene il gene modificato, che va a sostituirsi a quello originario. La cellula staminale embrionale con il gene mutato viene fatta proliferare in provetta. Nella fase successiva le cellule staminali embrionali così modificate vengono iniettate in un altro embrione di topo allo stadio iniziale dello sviluppo: a questo punto si forma un embrione chimera che viene inserito in una femmina; questa genera topi-chimera, che contengono solo in alcune cellule il gene modificato. I topolini-chimera si fanno accoppiare e generano così, in base alle leggi dell'ereditarietà, alcuni topolini sani e altri che hanno il gene mutato in tutte le cellule.



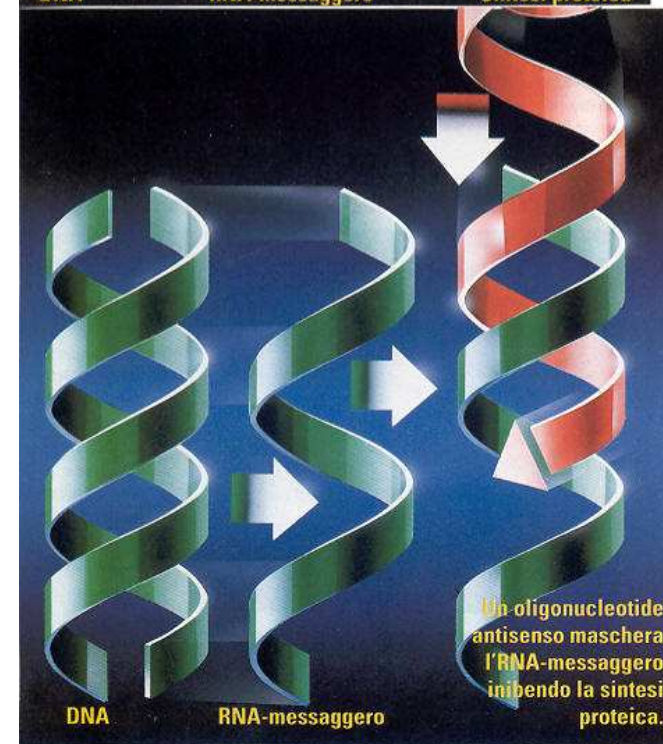
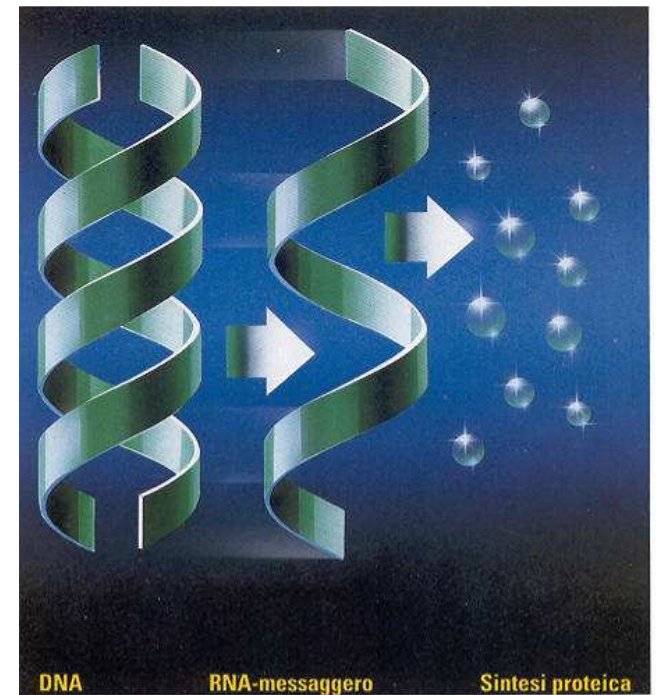
Topi KO (KNOCK OUT) (KO mice)



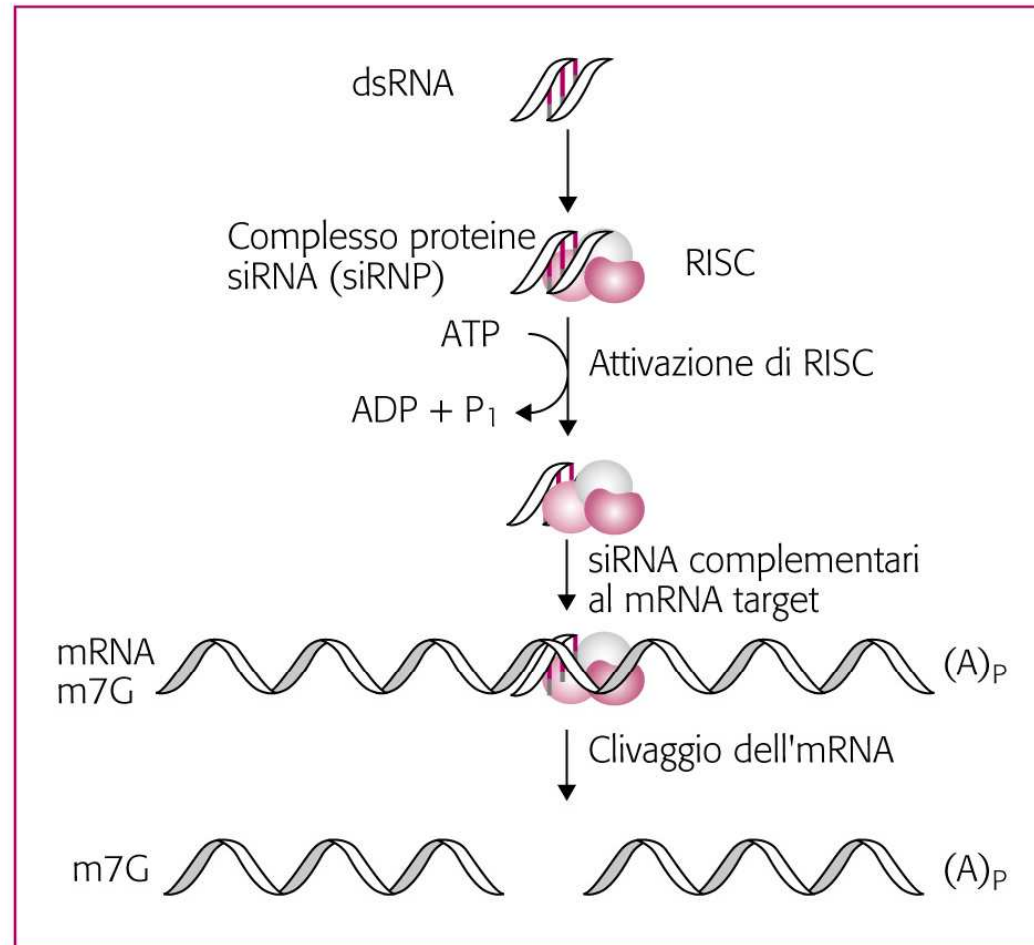
# Inibizione mirata dell'espressione genica

(es TG antisenso, RNA interference)

## TERAPIA GENICA “ANTISENSE”



## Trascritti non codificanti (ncRNA) e RNAi



**Figura 11.14** Silenziamento genico mediante siRNA. La presenza di piccoli RNA a doppio filamento, specifici per un dato mRNA, induce l'attivazione del complesso RISC, con formazione di siRNP (Small Interfering Ribonucleotidic Protein). Questo complesso si lega a un mRNA specifico e ne determina la degradazione mediante attività eso-endonucleasica.

# RNA interference

## Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs

Jürgen Soutschek<sup>1</sup>, Akin Akino<sup>2</sup>, Birgit Bramlage<sup>1</sup>, Klaus Charisse<sup>2</sup>, Rainer Constien<sup>1</sup>, Mary Donoghue<sup>2</sup>, Sayda Elbashir<sup>2</sup>, Anke Geick<sup>1</sup>, Philipp Hadwiger<sup>1</sup>, Jens Harborth<sup>1</sup>, Matthias John<sup>1</sup>, Venkatasamy Kesavan<sup>2</sup>, Gary Lavine<sup>2</sup>, Rajendra K. Pandey<sup>2</sup>, Timothy Racie<sup>2</sup>, Kallanthottathil G. Rajeev<sup>2</sup>, Ingo Röhl<sup>1</sup>, Ivanka Toudjarska<sup>2</sup>, Gang Wang<sup>2</sup>, Silvio Wuschko<sup>2</sup>, David Bumcrot<sup>2</sup>, Victor Kotliansky<sup>2</sup>, Stefan Limmer<sup>1</sup>, Muthiah Manoharan<sup>2</sup> & Hans-Peter Vornlocher<sup>1</sup>

<sup>1</sup>Ablynx Europe AG, Fritz-Hornschuch-Str. 9, 95326 Kulmbach, Germany

<sup>2</sup>Ablynx Pharmaceuticals Inc., 300 3rd Street, Cambridge, Massachusetts 02142, USA

news and views

## A cholesterol connection in RNAi

John J. Rossi

RNA interference — RNAi for short — might provide a way to silence disease-associated genes, but problems of delivery have hampered progress. Those problems may have been solved, at least in animal studies.

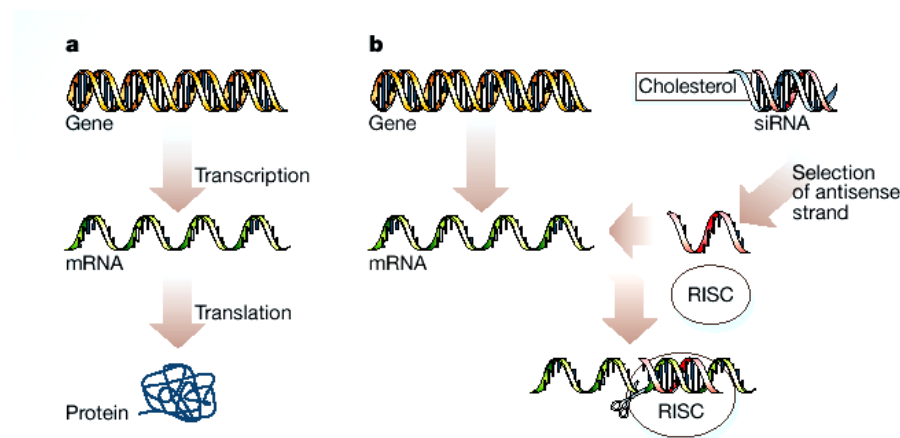


Figure 1 Silencing genes the RNAi way. a, For a gene to be expressed, its DNA sequence must be copied (transcribed) into messenger RNA (mRNA); this must in turn be translated into a protein sequence. b, RNAi works by either destroying the mRNA (bottom) or preventing it from being translated (not shown). In Soutschek and colleagues' modification<sup>1</sup> of the general RNAi approach, short interfering RNAs (siRNAs) are synthesized, chemically modified and labelled on the 'sense' strand (blue) with cholesterol. The siRNAs are then injected intravenously into mice, where the cholesterol group enables the siRNAs to be taken up into tissues. There, the sense strand is destroyed by the inherent RNAi pathway, leaving the antisense strand (red) to bind to a complementary sequence in a target mRNA. Recruitment of a protein complex, the RNA-induced silencing complex (RISC), enables the mRNA to be cleaved.

## Nobel per la Medicina 2006 e 2007: silenziamento genico e “gene targeting”



### Nobel Medicina: la molecola che sa spegnere i geni

I ricercatori americani Andrew Z. Fire e Craig C. Mello sono stati insigniti del prestigioso riconoscimento per le loro scoperte sull'informazione genetica



## Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*

Andrew Fire\*, SiQun Xu\*, Mary K. Montgomery\*, Steven A. Kostas\*†, Samuel E. Driver‡ & Craig C. Mello‡

\* Carnegie Institution of Washington, Department of Embryology, 115 West University Parkway, Baltimore, Maryland 21210, USA

† Biology Graduate Program, Johns Hopkins University, 3400 North Charles Street, Baltimore, Maryland 21218, USA

‡ Program in Molecular Medicine, Department of Cell Biology, University of Massachusetts Cancer Center, Two Biotech Suite 213, 373 Plantation Street, Worcester, Massachusetts 01605, USA

Experimental introduction of RNA into cells can be used in certain biological systems to interfere with the function of an endogenous gene<sup>1,2</sup>. Such effects have been proposed to result from a simple antisense mechanism that depends on hybridization between the injected RNA and endogenous messenger RNA transcripts. RNA interference has been used in the nematode *Caenorhabditis elegans* to manipulate gene expression<sup>3,4</sup>. Here we investigate the requirements for structure and delivery of the interfering RNA. To our surprise, we found that double-stranded RNA was substantially more effective at producing interference than was either strand individually. After injection into adult animals, purified single strands had at most a modest effect, whereas double-stranded mixtures caused potent and specific interference. The effects of this interference were evident in both the injected animals and their progeny. Only a few molecules of injected double-stranded RNA were required per affected cell, arguing against stoichiometric interference with endogenous

# 2006 Nobel Prize for Medicine





*Martedì, 25 novembre*

9.00 → 10.30      Sessioni Parallele – Comunicazioni orali

10.30 → 11.00      *Coffee break*

11.00 → 12.30      Sessione Plenaria  
**SILENZIAMENTO DELL'RNA**  
Moderatori: Roberto Ravazzolo (Genova, I)  
Maria Cristina Rosatelli (Cagliari, I)

11.00                  RNA non codificanti e regolazione  
dell'espressione genica  
Elisa Caffarelli (Roma, I)

11.30                  MicroRNA e tumorigenesi: diagnosi, prognosi  
e terapia  
Massimo Negrini (Ferrara, I)

12.00                  Utilizzo dei microRNA a scopo terapeutico  
Luigi Naldini (Milano, I)

## ARTICLES

# Mesoangioblast stem cells ameliorate muscle function in dystrophic dogs

Maurilio Sampaolesi<sup>1,2\*</sup>, Stéphane Biot<sup>3\*</sup>, Giuseppe D'Antona<sup>2</sup>, Nicolas Granger<sup>3</sup>, Rossana Tonlorenzi<sup>1</sup>, Anna Innocenzi<sup>1</sup>, Paolo Mogno<sup>4</sup>, Jean-Laurent Thibaud<sup>3</sup>, Beatriz G. Galvez<sup>1</sup>, Ines Barthélemy<sup>3</sup>, Laura Perani<sup>1</sup>, Sara Mantero<sup>4</sup>, Maria Guttinger<sup>5</sup>, Orietta Pansarasa<sup>2</sup>, Chiara Rinaldi<sup>2</sup>, M. Gabriella Cusella De Angelis<sup>2</sup>, Yvan Torrente<sup>6</sup>, Claudio Bordignon<sup>1</sup>, Roberto Bottinelli<sup>2</sup> & Giulio Cossu<sup>1,2,7</sup>

Duchenne muscular dystrophy remains an untreatable genetic disease that severely limits motility and life expectancy in affected children. The only animal model specifically reproducing the alterations in the dystrophin gene and the full spectrum of human pathology is the golden retriever dog model. Affected animals present a single mutation in intron 6, resulting in complete absence of the dystrophin protein, and early and severe muscle degeneration with nearly complete loss of motility and walking ability. Death usually occurs at about 1 year of age as a result of failure of respiratory muscles. Here we report that intra-arterial delivery of wild-type canine mesoangioblasts (vessel-associated stem cells) results in an extensive recovery of dystrophin expression, normal muscle morphology and function (confirmed by measurement of contraction force on single fibres). The outcome is a remarkable clinical amelioration and preservation of active motility. These data qualify mesoangioblasts as candidates for future stem cell therapy for Duchenne patients.

Duchenne muscular dystrophy primarily affects skeletal muscle, causing fibre degeneration, progressive paralysis and death<sup>1</sup>. No effective treatment exists although novel therapeutic strategies, ranging from new drugs to gene and cell therapy, hold promise for significant advance in the future<sup>2</sup>. In particular, different types of stem cell have been shown to induce dystrophin synthesis and partial rescue of the pathology in dystrophic mice<sup>3–6</sup>. However, dystrophic mice do not display clinical signs of the disease, and to proceed to a clinical trial it is imperative to show efficacy in a large, non-syngeneic animal model of muscular dystrophy. Golden retriever muscular dystrophy (GRMD)<sup>7,8</sup> is a very severe form of dystrophy, which

affects not only limb, respiratory and heart muscles but also pharyngeal muscles, resulting in a severe involvement of the digestive tract, although variability exists between individuals, by 8 months of age most dogs walk with great difficulty (Supplementary Movie 1). To test the efficacy of cell or gene therapy, we transplanted GRMD dogs with either autologous genetically corrected or donor wild-type mesoangioblasts, under different regimes of immune suppression.

Ten dystrophic dogs were treated in three experiments and a general scheme of treatments and outcome is reported in Table 1. Four dogs received autologous mesoangioblasts, transduced *in vitro* with a lentiviral vector expressing human microdystrophin (Supplementary

Table 1 | Summary of treatment

Dog no.	Dog name	Cell treatment	Lentiviral vector	Onset of treatment	Immune suppression (days)	Dystrophin expression	Motility	Outcome of experiment (at time P4 00)
01A	Ucal	Autologous, gene therapy	CK-pdys4-neo-GFP	P118	–	+/–	Loss	Euthanasia (P272)
02H	Vrlie	Heterologous, WT donor	–	P80	CYC A (P78)	+	Loss	Euthanasia (P235)
03H	Valgas	Heterologous, WT donor	–	P75	CYC A (P73)	+++	No decline	Alive and well
04H	Varus	Heterologous, WT donor	–	P75	RAP (P73)	+++	Modest decline	Alive and well
05H	Vlko	Heterologous, WT donor	–	P77	RAP + IL-10 (P74)	ND	ND (sudden death)	Myocarditis (P186)
06A	Vaccin	Autologous, gene therapy	MLC1F-pdys	P113	–	++	Major decline	Euthanasia (P326)
07A	Valum	Autologous, gene therapy	MLC1F-pdys	P113	–	ND	Loss	Pneumonia (P245)
08A	Vampine	Autologous, gene therapy	MLC1F-pdys	P113	–	++	Major decline	Pneumonia (P154)
09H	Azur	Heterologous, WT donor	–	P159	CYC A (P157)	++	Restored	Alive and well
10H	Azur	Heterologous, WT donor	–	P159	CYC A (P157)	+++	Restored	Alive and well
11U	Alan	None	–	–	–	–	Loss	Euthanasia (P380)
12U	Vulcano	None	–	–	–	–	Loss	Euthanasia (P376)
13U	Viking	None	–	–	–	–	Loss	Euthanasia (P340)

Each dog was given an official name and a sequential number, followed by A (transplanted with autologous cells), H (transplanted with heterologous cells) or U (untreated). The nature of the lentiviral vector is indicated: CK-pdys4-neo-GFP, control vector expressing driving microdystrophin-neo-GFP; MLC1F-pdys, myoD in light chain 1 (actin) promoter driving microdystrophin. Dystrophin expression was quantified as follows: –, average dystrophin (or microdystrophin) expression in less than 1% of positive fibres; +/–, less than 10% of positive fibres; ++, less than 50% of positive fibres; +++, more than 50% of positive fibres. CYC A, cyclosporine; RAP, rapamycin; WT, wild-type. Euthanasia was administered when clinical conditions worsened.

<sup>1</sup>San Raffaele Scientific Institute, Università Vita-Salute, Stem Cell Research Institute, Via Olgettina 58, 20132 Milan, Italy. <sup>2</sup>Department of Experimental Medicine and Interuniversity Institute of Myology, University of Pavia, Via Forlanini 6-B, 27100 Pavia, Italy. <sup>3</sup>Neurobiology laboratory, Ecole Vétérinaire d'Alfort, 7, Avenue Général de Gaulle, 94704 Maisons-Alfort cedex, France. <sup>4</sup>Department of Biotechnology, Politecnico di Milano, Piazza Leonardo da Vinci, 20133 Milan, Italy. <sup>5</sup>Institute of Cell Biology and Tissue Engineering, San Raffaele Biomedical Science Park of Roma, Via Castel Romano 100, 00120 Roma, Italy. <sup>6</sup>IRCCS Fondazione Policlinico di Milano, Department of Neurological Sciences, University of Milan, Via Sforza 35, 20122 Milan, Italy. <sup>7</sup>Department of Biology and Centre for Stem Cell Research, University of Milan, Via Celoria 28, 20130 Milan, Italy.

\*These authors contributed equally to this work.

## Cellule staminali e distrofia muscolare

## NEWS

---

Published online: 15 November 2006; |  
doi:10.1038/news061113-13

### **Stem cells treat wasted muscles**

**Dogs with muscular dystrophy walk better after injections.**

**Helen Pearson**

---

An infusion of stem cells scraped from blood vessels has helped dogs with a form of muscular dystrophy to walk more normally, perhaps heralding a treatment for the human disease.

Muscular dystrophies are a group of widespread genetic disorders in which the muscles gradually break down. The most common form, called Duchenne's



Golden retriever dogs



# Emofilia.....

## **Long term correction of inhibitor prone hemophilia B dogs treated with liver-directed AAV2 mediated factor IX gene therapy.**

[Niemeyer GP](#), [Herzog RW](#), [Mount J](#), [Arruda VR](#), [Tillson DM](#), [Hathcock J](#), [van Ginkel FW](#), [High KA](#), [Lothrop CD Jr](#).

Scott-Ritchey Research Center, College of Veterinary Medicine, Auburn University, Auburn, AL, United States.

Preclinical studies in mice, dogs and initial clinical trials have documented the feasibility of adeno-associated virus (AAV) mediated gene therapy for hemophilia B. In a 8 year study, inhibitor prone hemophilia B dogs (n=2) treated with liver directed AAV2 FIX gene therapy did not have a single bleed requiring FIX replacement; whereas, dogs undergoing muscle directed gene therapy (n=3) had a bleed frequency similar to untreated FIX deficient dogs. Coagulation tests (WBCT, ACT, APTT) have remained at the upper limits of the normal ranges in the two dogs which received liver directed gene therapy. The FIX activity has remained stable between 4-10% in both liver treated dogs but undetectable in the dogs undergoing muscle directed gene transfer. The vector/FIX sequences have persisted in liver biopsies but were undetectable in WBC and sperm DNA. Integration site analysis by LAM-PCR suggested the vector sequences have persisted predominantly in extrachromosomal form. A complete clinical evaluation of the dogs undergoing liver directed gene therapy including CBC, serum chemistries, bile acid profile, hepatic MRI and CT scans and liver biopsy was normal with no evidence for tumor formation. AAV mediated liver directed gene therapy corrected the hemophilia phenotype without toxicity or inhibitor development in the inhibitor prone null mutation dogs for more than 8 years.

## **The treatment of hemophilia A: from protein replacement to AAV-mediated gene therapy.**

[Youjin S](#), [Jun Y](#).

Department of Hematology, The Second Hospital of Shantou University Medical College, 515041, Shantou, China, shenyoujin@yahoo.com.cn.

Factor VIII (FVIII) is an essential component in blood coagulation, a deficiency of which causes the serious bleeding disorder hemophilia A. Recently, with the development of purification level and recombinant techniques, protein replacement treatment to hemophiliacs is relatively safe and can prolong their life expectancy. However, because of the possibility of unknown contaminants in plasma-derived FVIII and recombinant FVIII, and high cost for hemophiliacs to use these products, gene therapy for hemophilia A is an attractive alternative to protein replacement therapy. Thus far, the adeno-associated virus (AAV) is a promising vector for gene therapy. Further improvement of the virus for clinical application depends on better understanding of the molecular structure and fate of the vector genome. It is likely that hemophilia will be the first genetic disease to be cured by somatic cell gene therapy.

## **Factoring nonviral gene therapy into a cure for hemophilia A.**

[Gabrovsky V](#), [Calos MP](#).

Stanford University School of Medicine, Department of Genetics, Stanford, CA 94305-5120, USA.

Gene therapy for hemophilia A has fallen short of success despite several clinical trials conducted over the past decade. Challenges to its success include vector immunogenicity, insufficient transgene expression levels of Factor VIII, and inhibitor antibody formation. Gene therapy has been dominated by the use of viral vectors, as well as the immunogenic and oncogenic concerns that accompany these strategies. Because of the complexity of viral vectors, the development of nonviral DNA delivery methods may provide an efficient and safe alternative for the treatment of hemophilia A. New types of nonviral strategies, such as DNA integrating vectors, and the success of several nonviral animal studies, suggest that nonviral gene therapy has curative potential and justifies its clinical development.

# Distrofie muscolari.....

1: [Curr Gene Ther.](#) 2008 Oct;8(5):391-405.

## Muscular gene transfer using nonviral vectors.

[Braun S.](#)

AFM (Association Française contre les Myopathies), 1 rue de l'Internationale, 91002 EVRY Cedex, France. [sbraun@afm.genethon.fr](mailto:sbraun@afm.genethon.fr).

Skeletal muscle is a target tissue of choice for the gene therapy of both muscle and non-muscle disorders. Investigations of gene transfer into muscle have progressed considerably from the expression of plasmid reporter genes to the production of therapeutic proteins such as trophic factors, hormones, antigens, ion channels or cytoskeletal proteins. Viral vectors are intrinsically the most efficient vehicles to deliver genes into skeletal muscles. But, because viruses are associated with a variety of problems (such as immune and inflammatory responses, toxicity, limited large scale production yields, limitations in the size of the carried therapeutic genes), nonviral vectors remain a viable alternative. In addition, as nonviral vectors allow to transfer genetic structures of various sizes (including large plasmid DNA carrying full-length coding sequences of the gene of interest), they can be used in various gene therapy approaches. However, given the lack of efficiency of nonviral vectors in experimental studies and in the clinical settings, the overall outcome clearly indicates that improved synthetic vectors and/or delivery techniques are required for successful clinical gene therapy. Today, most of the potential muscle-targeted clinical applications seem geared toward peripheral ischemia (mainly through local injections) and cancer and infectious vaccines, and one locoregional administration of naked DNA in Duchenne muscular dystrophy. This review updates the developments in clinical applications of the various plasmid-based non-viral methods under investigation for the delivery of genes to muscles.

1: [Mol Ther.](#) 2008 Oct 21. [Epub ahead of print]

## Transduction Efficiency and Immune Response Associated With the Administration of AAV8 Vector Into Dog Skeletal Muscle.

[Ohshima S](#), [Shin JH](#), [Yuasa K](#), [Nishiyama A](#), [Kira J](#), [Okada T](#), [Takeda S](#).

[1] 1Department of Molecular Therapy, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Tokyo, Japan [2] 2Department of Neurology, Neurological Institute, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan.

Recombinant adeno-associated virus (rAAV)-mediated gene transfer is an attractive approach to the treatment of Duchenne muscular dystrophy (DMD). We investigated the muscle transduction profiles and immune responses associated with the administration of rAAV2 and rAAV8 in normal and canine X-linked muscular dystrophy in Japan (CXMD(J)) dogs. rAAV2 or rAAV8 encoding the lacZ gene was injected into the skeletal muscles of normal dogs. Two weeks after the injection, we detected a larger number of beta-galactosidase-positive fibers in rAAV8-transduced canine skeletal muscle than in rAAV2-transduced muscle. Although immunohistochemical analysis using anti-CD4 and anti-CD8 antibodies revealed less T-cell response to rAAV8 than to rAAV2, beta-galactosidase expression in rAAV8-injected muscle lasted for <4 weeks with intramuscular transduction. Canine bone marrow-derived dendritic cells (DCs) were activated by both rAAV2 and rAAV8, implying that innate immunity might be involved in both cases. Intravenous administration of rAAV8-lacZ into the hind limb in normal dogs and rAAV8-microdystrophin into the hind limb in CXMD(J) dogs resulted in improved transgene expression in the skeletal muscles lasting over a period of 8 weeks, but with a declining trend. The limb perfusion transduction protocol with adequate immune modulation would further enhance the rAAV8-mediated transduction strategy and lead to therapeutic benefits in DMD gene therapy. *Molecular Therapy* (2008); doi:10.1038/mt.2008.225.

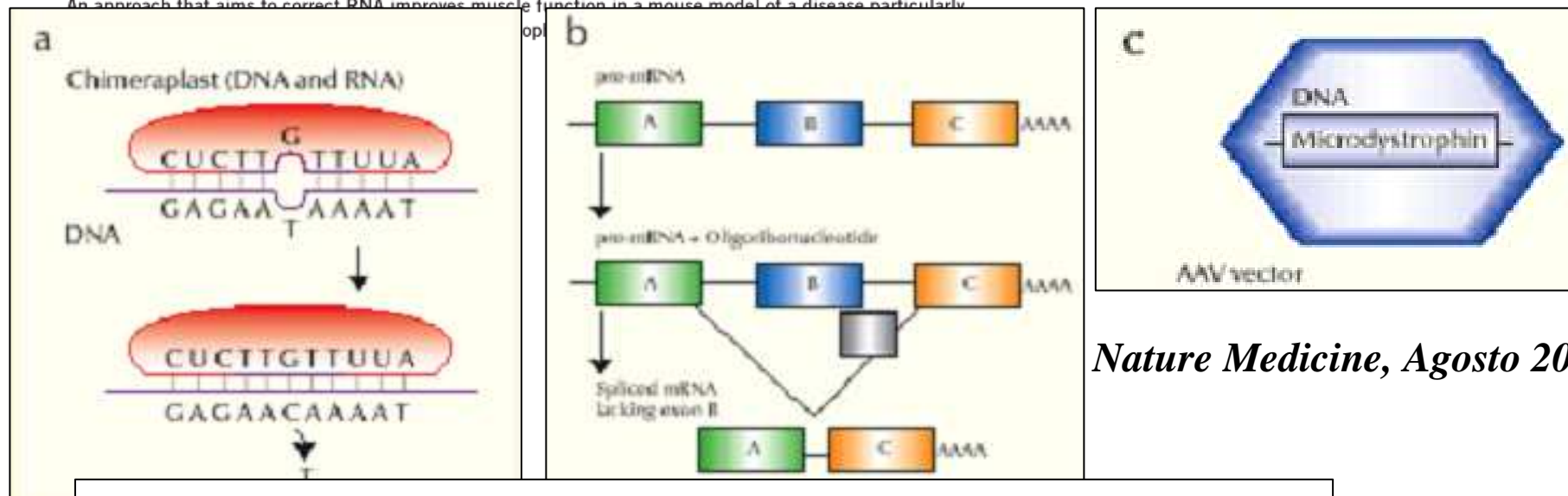
# TERAPIA GENICA SOMATICA

## DISTROFIA MUSCOLARE

### Skipping to new gene therapies for muscular dystrophy

James G Tidball & Melissa J Spencer

An approach that aims to correct RNA improves muscle function in a mouse model of a disease particularly



*Nature Medicine, Agosto 2003*

Functional amounts of dystrophin produced by skipping the mutated exon in the *mdx* dystrophic mouse

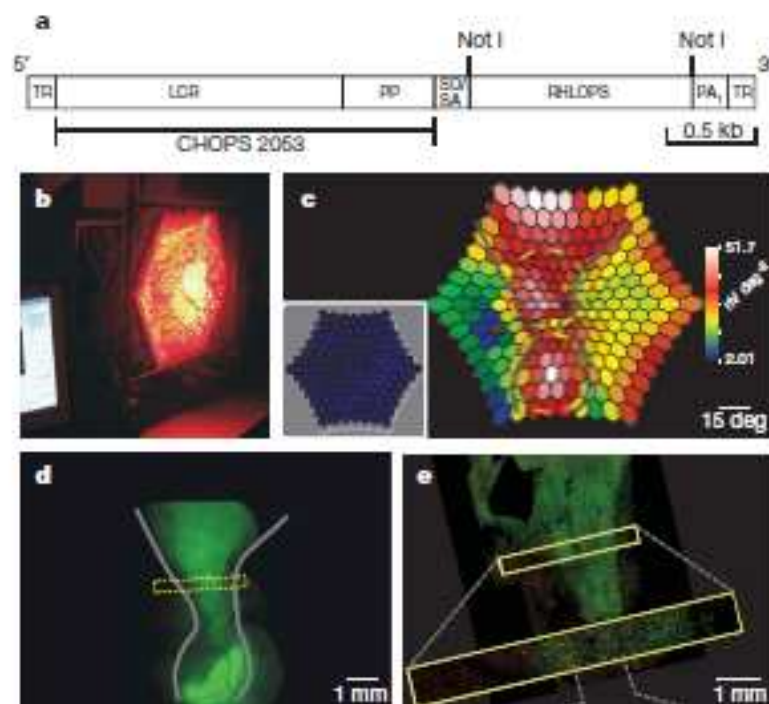
## LETTERS

## Gene therapy for red–green colour blindness in adult primates

Katherine Mancuso<sup>1</sup>, William W. Hauswirth<sup>2</sup>, Qihong Li<sup>2</sup>, Thomas B. Connor<sup>3</sup>, James A. Kuchenbecker<sup>1</sup>, Matthew C. Mauck<sup>3</sup>, Jay Neitz<sup>1</sup> & Maureen Neitz<sup>1</sup>

Red–green colour blindness, which results from the absence of either the long- (L) or the middle- (M) wavelength-sensitive visual photopigments, is the most common single locus genetic disorder. Here we explore the possibility of curing colour blindness using gene therapy in experiments on adult monkeys that had been colour blind since birth. A third type of cone pigment was added to dichromatic retinas, providing the receptor basis for trichromatic colour vision. This opened a new avenue to explore the requirements for establishing the neural circuits for a new dimension of colour sensation. Classic visual deprivation experiments<sup>1</sup> have led to the expectation that neural connections established during development would not appropriately process an input that was not present from birth. Therefore, it was believed that the treatment of congenital vision disorders would be ineffective unless administered to the very young. However, here we show that the addition of a third opsin in adult red–green colour-deficient primates was sufficient to produce trichromatic colour vision behaviour. Thus, trichromacy can arise from a single addition of a third cone class and it does not require an early developmental process. This provides a positive outlook for the potential of gene therapy to cure adult vision disorders.

Gene therapy was performed on adult squirrel monkeys (*Saimiri*





## NEWS &amp; VIEWS

## GENE THERAPY

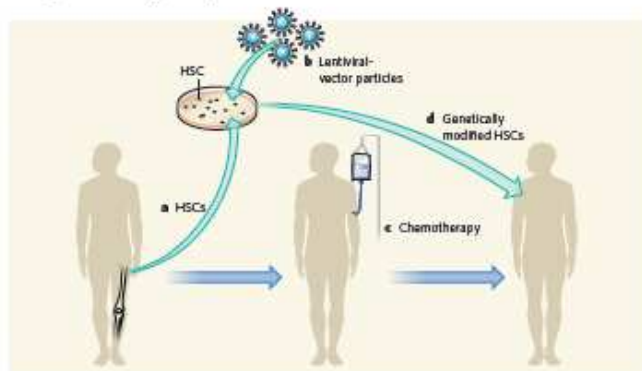
Targeting  $\beta$ -thalassaemia

Derek A. Persons

**Patients with disorders of the blood protein haemoglobin often depend on lifelong blood transfusions. That could change, given the success of gene therapy in a patient with one such disorder.**

$\beta$ -Thalassaemia is one of several inherited disorders associated with abnormalities in the oxygen-carrying protein haemoglobin. It is caused by mutations in the  $\beta$ -globin chain of haemoglobin that lead to ineffective production of red blood cells and profound anaemia. Patients with  $\beta$ -thalassaemia require regular blood transfusions for life. Chronic transfusions have a significant impact on the quality of life and ultimately shorten life expectancy. As for treating this disorder, until now the only available strategy has been the transplantation of bone-marrow cells, a procedure whose success depends on the availability of suitable donors. A therapy based on genetic correction of a patient's own bone-marrow cells has therefore long been awaited<sup>1</sup>. On page 318 of this issue, Cavazzana-Calvo *et al.*<sup>2</sup> deliver news of one such success story using gene therapy.

The potential of human gene therapy first became apparent about a decade ago. In clinical trials, children with inherited, life-threatening immune disorders were given their own pretreated bone-marrow haematopoietic stem



**Figure 1 | Gene-therapy procedure.** a, Cavazzana-Calvo *et al.*<sup>2</sup> collected haematopoietic stem cells (HSCs) from the bone marrow of a patient with  $\beta$ -thalassaemia and maintained them in culture. b, The authors then introduced lentiviral-vector particles containing a functional  $\beta$ -globin gene into the cells and allowed them to expand further in culture. c, To eradicate the patient's remaining HSCs and make room for the genetically modified cells, the patient underwent chemotherapy. d, The genetically modified HSCs were then transplanted into the patient.

## LETTERS

Transfusion independence and *HMGA2* activation after gene therapy of human  $\beta$ -thalassaemia

Marina Cavazzana-Calvo<sup>1,2\*</sup>, Emmanuel Payen<sup>3,4,5\*</sup>, Olivier Negre<sup>3,4,5,6</sup>, Gary Wang<sup>7</sup>, Kathleen Hehir<sup>8</sup>, Floriane Fusil<sup>1,4,5</sup>, Julian Down<sup>9</sup>, Maria Denaro<sup>9</sup>, Troy Brady<sup>9</sup>, Karen Westerman<sup>8,9</sup>, Resy Cavalleco<sup>9</sup>, Beatrix Gillet-Legrand<sup>9</sup>, Laure Caccavelli<sup>1,2</sup>, Riccardo Sgarra<sup>10</sup>, Leila Maouche-Chrétien<sup>1,4</sup>, Françoise Bernaudin<sup>1,1</sup>, Robert Giroi<sup>12</sup>, Ronald Dorazio<sup>9</sup>, Geert-Jan Mulder<sup>9</sup>, Axel Polack<sup>9</sup>, Arthur Bank<sup>13</sup>, Jean Soulier<sup>9</sup>, Jérôme Larghero<sup>9</sup>, Nabil Kabbara<sup>9</sup>, Bruno Dalle<sup>9</sup>, Bernard Goumel<sup>9</sup>, Gérard Socie<sup>9</sup>, Stany Chrétien<sup>3,4,9</sup>, Nathalie Cartier<sup>1,4</sup>, Patrick Aubourg<sup>1,4</sup>, Alain Fischer<sup>1,2</sup>, Kenneth Cornetta<sup>15</sup>, Frédéric Galacteros<sup>16</sup>, Yves Beuzard<sup>3,4,9</sup>, Eliane Gluckman<sup>9</sup>, Frederick Bushman<sup>7</sup>, Salima Hachein-Bey-Abina<sup>1,2\*</sup> & Philippe Leboulch<sup>3,4,9\*</sup>

The  $\beta$ -haemoglobinopathies are the most prevalent inherited disorders worldwide. Gene therapy of  $\beta$ -thalassaemia is particularly challenging given the requirement for massive haemoglobin production in a lineage-specific manner and the lack of selective advantage for corrected haematopoietic stem cells. Compound  $\beta^0/\beta^0$ -thalassaemia is the most common form of severe thalassaemia in southeast Asian countries and their diasporas<sup>1,2</sup>. The  $\beta^0$ -globin allele bears a point mutation that causes alternative splicing. The abnormally spliced form is non-coding, whereas the correctly spliced messenger RNA expresses a mutated  $\beta^0$ -globin with partial instability<sup>3</sup>. When this is compounded with a non-functional  $\beta^0$  allele, a profound decrease in  $\beta$ -globin synthesis results, and approximately half of  $\beta^0/\beta^0$ -thalassaemia patients are transfusion-dependent<sup>2</sup>. The only available curative therapy is allogeneic haematopoietic stem cell transplantation, although most patients do not have a human-leukocyte-antigen-matched, gene-identical donor, and those who do still risk rejection or graft-versus-host disease. Here we show that, 35 months after lentiviral  $\beta$ -globin gene transfer, an adult patient with severe  $\beta^0/\beta^0$ -thalassaemia dependent on monthly transfusions since early childhood has become transfusion independent for the past 21 months. Blood haemoglobin is maintained between 9 and 10 g dL<sup>-1</sup>, of which one-third contains vector-encoded  $\beta$ -globin. Most of the therapeutic benefit results from a dominant, myeloid-biased cell clone, in which the integrated vector causes transcriptional activation of *HMGA2* in erythroid cells with further increased expression of a truncated *HMGA2* mRNA insensitive to degradation by let-7 microRNAs. The clonal dominance that accompanies therapeutic efficacy may be coincidental and stochastic or result from a hitherto benign cell expansion caused by dysregulation of the *HMGA2* gene in stem/progenitor cells.

fidelity and high titres<sup>4,5</sup>. Hence, several mouse models of the  $\beta$ -haemoglobinopathies have been corrected, long-term, by *ex vivo* transduction of haematopoietic stem cells (HSCs) with  $\beta$ -globin lentiviral vectors<sup>6–10</sup>. These advances have prompted the prudent initiation of a human clinical trial (Supplementary Note 1).

The general structure of the  $\beta$ -globin-expressing lentiviral vector has been previously described<sup>6,11</sup> (Supplementary Fig. 1). It is a self-inactivating vector with two copies of the 250-base-pair (bp) core of the cHS4 chromatin insulator<sup>12</sup> implanted in the U3 region. It encodes a mutated adult  $\beta$ -globin ( $\beta^0$ ) with anti-silencing properties<sup>13</sup> that can be distinguished from normal adult  $\beta$ -globin ( $\beta^+$ ) by high-performance liquid chromatography (HPLC) analysis in individuals receiving red blood cell transfusions and/or  $\beta^0$ -thalassaemia patients.

This report focuses on the first treated patient (P2) who did not receive back-up cells: a male, aged 18 years at the time of treatment, with severe  $\beta^0/\beta^0$ -thalassaemia. A previous patient (P1) failed to engraft because the HSCs had been compromised by the technical handling of the cells without relation to the gene therapy vector. P1 failed to engraft after 5 weeks and was thus given back-up cells (Supplementary Note 2). P2 was first transfused at age three because of poorly tolerated anaemia (6.7 g dL<sup>-1</sup> despite residual fetal haemoglobin (HbF)) and major hepatosplenomegaly. Transfusion requirements rapidly increased to once a month (2–3 red blood cell packs each time; 157 ml of red blood cells per kg the year before transplant). He was splenectomized at age 6. In spite of this, Hb levels decreased several times to as low as 4 g dL<sup>-1</sup>, and hydroxyurea therapy was ineffective. Iron chelation was initiated at age 8 by parenteral deferoxamine overnight, 5 times a week. The patient did not have a related human-leukocyte-antigen-matched donor and was thus enrolled in this trial after informed consent.

# TERAPIA GENICA

## PROBLEMI PRINCIPALI

- **Espressione genica bassa o transitoria**
- **Ridotte dimensioni dell'inserito ("gene terapeutico")**
- **Difficoltà a raggiungere alcuni tessuti (es. SNC)**
- **Risposte immunitarie nell'ospite**
- **Difficoltà ad ottenere una precisa regolazione dell'espressione genica**
- **Incapacità ad infettare cellule quiescenti (es. retrovirus)**
- **Potenziabile ruolo oncogeno (mutagenesi da inserzione)**

## Terapia genica “migliorativa” (genetic enhancement)

Il doping dei geni cambierà la natura dello sport

# Atleti geneticamente modificati

- Quando il metano dominava il clima
- Codice Voynich: truffa o mistero?
- Rane, parassiti e nuove malattie

POSTE ITALIANE SPEED IN A.P. - D.L. 353/2003 CONV. L. 46/2004, ART. 1, C. 1, DDB - MILANO Numero 432 - agosto 2004 - € 3,90







**La Consulta di Bioetica - Sezione di Verona e Sezione di Pisa  
con il patrocinio dell'Università degli Studi di Verona**

*presentano un convegno sul tema*

**POTENZIAMENTO BIOLOGICO  
*ENHANCEMENT* e QUESTIONI ETICHE**



**venerdì 11 dicembre 2009 - ore 15.00**

**Aula Magna della Facoltà di Medicina e Chirurgia  
Università degli Studi di Verona  
Policlinico G.B. Rossi - Piazzale L.A. Scuro, 10 - Borgo Roma - Verona**

**INTERVENGONO**

*Prof.ssa Sara Patuzzo*

Consulta di Bioetica - Sezione di Verona

*Prof. Alberto Turco*

Consulta di Bioetica - Sezione di Verona

*Prof. Roberto Leone*

Università di Verona

*Prof. Cristiano Chiamulera*

Università di Verona

*Prof. Paolo Fiorini*

Università di Verona

*Prof. Roberto Foroni*

Università di Verona

**M O D E R A**

*Prof. Sergio Bartolommei*

Consulta di Bioetica - Sezione di Pisa

[www.consultadibioetica.org](http://www.consultadibioetica.org)



# Xenotransplantation.....

## Production of $\alpha$ -1,3-Galactosyltransferase Knockout Pigs by Nuclear Transfer Cloning

Liangxue Lai,<sup>1</sup> Donna Kolber-Simonds,<sup>3</sup> Kwang-Wook Park,<sup>1</sup> Hee-Tae Cheong,<sup>1,4</sup> Julia L. Greenstein,<sup>3</sup> Gi-Sun Im,<sup>1,5</sup> Melissa Samuel,<sup>1</sup> Aaron Bonk,<sup>1</sup> August Rieke,<sup>1</sup> Billy N. Day,<sup>1</sup> Clifton N. Murphy,<sup>1</sup> David B. Carter,<sup>1,2</sup> Robert J. Hawley,<sup>3</sup> Randall S. Prather<sup>1\*</sup>



The presence of galactose  $\alpha$ -1,3-galactose residues on the surface of pig cells is a major obstacle to successful xenotransplantation. Here, we report the production of four live pigs in which one allele of the  $\alpha$ -1,3-galactosyltransferase locus has been knocked out. These pigs were produced by nuclear transfer technology; clonal fetal fibroblast cell lines were used as nuclear donors for embryos reconstructed with enucleated pig oocytes.



## Production of $\alpha$ 1,3-Galactosyltransferase-Deficient Pigs

Carol J. Phelps,<sup>1</sup> Chihiro Koike,<sup>3,4</sup> Todd D. Vaught,<sup>1</sup> Jeremy Boone,<sup>1</sup> Kevin D. Wells,<sup>1</sup> Shu-Hung Chen,<sup>1</sup> Suyapa Ball,<sup>1</sup> Susan M. Specht,<sup>3,4</sup> Irina A. Polejaeva,<sup>1</sup> Jeff A. Monahan,<sup>1</sup> Pete M. Jobst,<sup>1</sup> Sugandha B. Sharma,<sup>3,4</sup> Ashley E. Lamborn,<sup>1</sup> Amy S. Garst,<sup>1</sup> Marilyn Moore,<sup>2</sup> Anthony J. Demetris,<sup>3,5</sup> William A. Rudert,<sup>3,6</sup> Rita Bottino,<sup>3,6</sup> Suzanne Bertera,<sup>3,6</sup> Massimo Trucco,<sup>3,6</sup> Thomas E. Starzl,<sup>3,4</sup> Yifan Dai,<sup>1\*</sup> David L. Ayares<sup>1\*</sup>

The enzyme  $\alpha$ 1,3-galactosyltransferase ( $\alpha$ 1,3GT or GGTA1) synthesizes  $\alpha$ 1,3-galactose ( $\alpha$ 1,3Gal) epitopes (Gal $\alpha$ 1,3Gal $\beta$ 1,4GlcNAc-R), which are the major xenoantigens causing hyperacute rejection in pig-to-human xenotransplantation. Complete removal of  $\alpha$ 1,3Gal from pig organs is the critical step toward the success of xenotransplantation. We reported earlier the targeted disruption of one allele of the  $\alpha$ 1,3GT gene in cloned pigs. A selection procedure based on a bacterial toxin was used to select for cells in which the second allele of the gene was knocked out. Sequencing analysis demonstrated that knockout of the second allele of the  $\alpha$ 1,3GT gene was caused by a T-to-G single point mutation at the second base of exon 9, which resulted in inactivation of the  $\alpha$ 1,3GT protein. Four healthy  $\alpha$ 1,3GT double-knockout female piglets were produced by three consecutive rounds of cloning. The piglets carrying a point mutation in the  $\alpha$ 1,3GT gene hold significant value, as they would allow production of  $\alpha$ 1,3Gal-deficient pigs free of antibiotic-resistance genes and thus have the potential to make a safer product for human use.

The enzyme  $\alpha$ 1,3-galactosyltransferase ( $\alpha$ 1,3GT or GGTA1) synthesizes  $\alpha$ 1,3Gal epitopes (Gal $\alpha$ 1,3Gal $\beta$ 1,4GlcNAc-R) on the cell surface of almost all mammals with the exception of humans, apes, and Old World monkeys (1).  $\alpha$ 1,3Gal epitopes are the major xenoantigens causing hyperacute rejection (HAR) in pig-to-human xenotransplantation (2-4). Many reports have also indicated that

$\alpha$ 1,3Gal epitopes are involved in acute vascular rejection (AVR) of xenografts (4-6). Piglets with  $\alpha$ 1,3GT heterozygous knockout have been cloned by our group (7) and another team (8) in the last year. To produce homozygous  $\alpha$ 1,3GT knockout piglets by natural breeding, assuming both male and female heterozygous knockout pigs are available at the same time and are fertile, is feasible but takes up to 12 months. However, by using a second-round knockout and cloning strategy, we could save up to 6 months and all cloned piglets would be  $\alpha$ 1,3GT double knockout (DKO). We have selected and enriched for  $\alpha$ 1,3GT DKO cells by using a bacterial toxin, toxin A from *Clostridium difficile*, which binds with high affinity to  $\alpha$ 1,3Gal epitopes and produces a cytotoxic effect on cells that are  $\alpha$ 1,3Gal-positive (9). Toxin A uses  $\alpha$ 1,3Gal epitopes as a cell

<sup>1</sup>PPL Therapeutics Inc., 1700 Kraft Drive, Blacksburg, VA 24060, USA. <sup>2</sup>PPL Therapeutics Ltd., Roslin, Midlothian, EH25 9PP, UK. <sup>3</sup>Thomas E. Starzl Transplantation Institute, <sup>4</sup>Department of Surgery, <sup>5</sup>Department of Pathology, and <sup>6</sup>Department of Pediatrics (Division of Immunogenetics) of University of Pittsburgh Medical Center (UPMC) and Children's Hospital, Pittsburgh, PA 15213, USA.

\*To whom correspondence should be addressed. E-mail: ydai@ppl-therapeutics.com; dayares@ppl-therapeutics.com

# TRAPIANTO

Auto

stesso individuo

Iso

Allo

{ stessa specie  
individui diversi }

{ MZ

{ geneticamente  
diversi }

Xeno

specie diverse

# **XENOTRAPIANTO DI RENE MAIALE- SCIMMA.....**

Production of  
 $\alpha$ 1,3-Galactosyltransferase-  
Deficient Pigs



**Engineered pig organs survive in monkeys**

Humanised kidneys appear to thwart first round of rejection

*Dicembre 2003*

- Nonostante la grande mole di studi e ricerche, e le promesse iniziali, ad oggi la TG (classica) non ha mostrato applicazioni cliniche efficaci se non in pochi casi di ADA-SCID
- Esistono tuttora ostacoli e rischi, come la scarsa espressione genica, l'immunogenicità virale, e la possibile mutagenicità da inserzione
- Prospettive: terapia cellulare, iPS (cellule staminali indotte)